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14. ABSTRACT Research was conducted to demonstrate real-time detection and monitoring of foodborne pathogens and transgenes within buffers, foods and plants. Signature molecules derived from the Gram-positive bacterium, <i>Bacillus thuringiensis</i> , transgenic tobacco containing the transgene, Bt cry1Ac, the Gram-negative bacterium, <i>Salmonella Typhimurium</i> , and the Gram-positive bacterium, <i>Listeria monocytogenes</i> , were monitored for detection by coupling molecular beacon (MB) technology utilizing fluorescent resonance energy transfer (FRET), luminescent					
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Report Title

Biomolecular architectures molecular biology

ABSTRACT

Research was conducted to demonstrate real-time detection and monitoring of foodborne pathogens and transgenes within buffers, foods and plants. Signature molecules derived from the Gram-positive bacterium, *Bacillus thuringiensis*, transgenic tobacco containing the transgene, Bt cry1Ac, the Gram-negative bacterium, *Salmonella Typhimurium*, and the Gram-positive bacterium, *Listeria monocytogenes*, were monitored for detection by coupling molecular beacon (MB) technology utilizing fluorescent resonance energy transfer (FRET), luminescent nanoscale semiconductor quantum dots and quenchers. Probes were designed and prepared for detecting the Bt cry1Ac gene, the *invA* gene, and the *hlyA* gene from *B. thuringiensis*, *S. Typhimurium* and *L. monocytogenes* respectively, for use in real-time monitoring within buffer, water, milk, apple juice, chicken broth, and *Arabidopsis* leaves. We successfully detected changes in fluorescence at MB concentrations from ca. 1.2 to 40 nM, depending upon respective target and target concentration. We demonstrated the ability to detect the presence of target sequence and nucleic acid extracted from bacteria (in vitro monitoring) in several liquid food systems and within plant tissue, which demonstrates use in monitoring for bacterial pathogens in foods and crops.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
08/28/2012	3.00 Kellie P. Burris, C. Neal Stewart. Fluorescent nanoparticles: Sensing pathogens and toxins in foods and crops, Trends in Food Science & Technology, (07 2012): 0. doi: 10.1016/j.tifs.2012.06.013
TOTAL:	1

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

<u>Received</u>	<u>Paper</u>
08/22/2011	1.00 Milana Vasudev, Tsai-Chin Wu, Sushmita Biswas, Mitra Dutta, M. Stroschio, Stan Guthrie, Mark Reed, K. Burris, C. Neal Stewart. Optoelectronic Signatures of DNA-Based Hybrid Nanostructures, IEEE Transactions on Nanotechnology, (01 2011): 0. doi: 10.1109/TNANO.2010.2064174
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Number of Papers published in non peer-reviewed journals:

(c) Presentations

Burris, K.P., T-C. Wu, M. Stroschio, M. Vasudev, R. Millwood, and C. N. Stewart, Jr. 2011. Rapid detection and monitoring of bacterial pathogens and transgenes using molecular beacon technology. Nanoelectronic Devices for Defense & Security Conference (August 29-September 1, 2011), Brooklyn, NY.

Burris, K.P., Reggie Millwood, and C. Neal Stewart, Jr. 2011. Biomolecular architectures molecular biology: Molecules for THz spectroscopy and molecular beacon technology. ARO-DTRA PIs project review meeting (July 13-14, 2011), Washington, DC. (invited).

Burris, K.P., M. Stroschio, T.-C. Wu, M. Vasudev, R.J. Millwood and C. Neal Stewart, Jr. 2010. Real-time mega-nano detection of foodborne pathogens and transgenes using molecular beacon technology. International Symposium on Spectral Sensing Research (June 21-24 2010), Springfield, MO. Abstract 210. (invited).

Globus, T., B. Gelmont, C. N. Stewart, Jr., R. J. Millwood, and T. Khromova. 2010. THz spectroscopic signatures from short artificial DNA molecules, Oral presentation. ISSSR 2010, Sprngfield, MO. The 2010 International Symposium for Spectral Sensing Research (ISSSR). Abstract 101.

Burris, K.P., M. Stroschio, M. Vasudev, D. Wu, and C. Neal Stewart, Jr. 2009. In vivo mega-nano detection of foodborne pathogens and transgenes using molecular beacon technology. Nanoelectronic Devices for Defense & Security Conference (Sept 28-Oct 2, 2009), Fort Lauderdale, FL. (invited).

Vasudev, M., T.-C. Wu, M. Dutta, M.A. Stroschio, S. Guthrie, M. Reed, K. Burris, and C. N. Stewart, Jr. 2009. Optoelectric signatures of DNA based hybrid nanostructures. Nanoelectronic Devices for Defense & Security Conference (Sept 28-Oct 2, 2009), Fort Lauderdale, FL.

Burris, K.P., and C. Neal Stewart, Jr. 2009. Mega-nano structure for detecting foodborne pathogens. ARO PIs project meeting (March 19-20, 2009), Raleigh NC. (invited).

Number of Presentations: 7.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

<u>Received</u>	<u>Paper</u>
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TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

<u>Received</u>	<u>Paper</u>
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TOTAL:

(d) Manuscripts

<u>Received</u>	<u>Paper</u>
03/27/2013	5.00 Weidong Zhang, Elliott R. Brown, Leamon Viveros, Kellie Burris, C. Neal Stewart, Jr.?. Narrow terahertz attenuation signatures in <i>Bacillus thuringiensis</i> , Journal of Biophotonics (03 2013)
08/26/2011	2.00 Kellie P. Burris , C. Neal Stewart, Jr.. Fluorescent nanoparticles: sensing pathogens and toxins in foods and crop, TRENDS in Food Science& Technology (SUBMITTED) (08 2011)
08/28/2012	4.00 Kellie Burris, Tsai-Chin Wu, Milana Vasudev, Michael Stroschio, Reginald Millwood, C. Neal Stewart, Jr.. Mega-Nano Detection of Foodborne Pathogens and Transgenes Using Molecular Beacon Technology and Semiconductor Quantum Dot Technologies , IEEE Transactions on NanoBioScience (07 2012)
TOTAL:	3

Number of Manuscripts:

Books

<u>Received</u>	<u>Paper</u>
TOTAL:	

Patents Submitted

Patents Awarded

Awards

Kellie Burris received the Best student poster award at the 2009 NANO-DDS Conference in Fort Lauderdale, FL, Sept 28-Oct 2, 2009.

Kellie Burris received a travel award to attend the 2010 ISSSR Conference during the week of June 21-24, 2010 in Springfield, MO for \$1050.00 kindly provided by the US Army Research Office (ARO) and North Carolina A&T University.

Graduate Students

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
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FTE Equivalent:

Total Number:

Names of Post Doctorates

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
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Kellie Burris	1.00
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FTE Equivalent: 1.00

Total Number: 1

Names of Faculty Supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>	National Academy Member
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Neal Stewart	0.05	
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FTE Equivalent: 0.05

Total Number: 1

Names of Under Graduate students supported

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
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FTE Equivalent:

Total Number:

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

<u>NAME</u>

Total Number:

Names of personnel receiving PhDs

<u>NAME</u>

Total Number:

Names of other research staff

<u>NAME</u>	<u>PERCENT SUPPORTED</u>
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Reggie Millwood	0.05
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FTE Equivalent:	0.05
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Total Number:	1
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Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See attachment.

Technology Transfer

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

Table of Contents

Table of Contents.....	1
Overall Project Goals.....	4
Research Goals	4
Achievements	5
Achievement #1: Title: - TERAHERTZ SPECTROSCOPIC SIGNATURE FROM SHORT ARTIFICIAL DNA MOLECULES WITH WELL CONTROLLED STRUCTURE	5
Description	5
Results	5
Presentations	5
Achievement #2: Title: - TERAHERTZ SPECTROSCOPIC SIGNATURE FROM BACILLUS THURINGIENSIS AND BACILLUS SUBTILIS	5
Description	5
Results	6
Publications	6
Achievement #3: Title: - MOLECULAR BEACON DESIGN	7
Description	7
Results	7
References	7
Achievement #4: Title: - MOLECULAR BEACON PRODUCTION	7
Description	7
Results	8
Achievement #5: Title: - USE OF MOLECULAR BEACONS FOR DETECTING FOODBORNE PATHOGENS, SALMONELLA TYPHIMURIUM AND LISTERIA MONOCYTOGENES AND INSECT PATHOGEN, BACILLUS THURINGIENSIS OR THE TRANSGENE, Bt CRY1Ac.....	8
Description	8
Results	9
Presentations	11
Publications	12
References	12
Appendix 1. Tables and figures for Achievement #1: Title: - TERAHERTZ SPECTROSCOPIC SIGNATURE FROM SHORT ARTIFICIAL DNA MOLECULES WITH WELL CONTROLLED STRUCTURE.	13
Table 1. Short synthetic 25 bp and 50 bp sequences.	13
Appendix 2. Tables and figures for Achievement #2: Title: - TERAHERTZ SPECTROSCOPIC SIGNATURE FROM BACILLUS THURINGIENSIS AND BACILLUS SUBTILIS.....	14
Table 1. Wet weights and enumeration of six samples of vegetative cells and endospores derived from the HD73-20 strain of <i>Bacillus thuringiensis</i> for THz spectroscopy measurements.	14
Figure 1. Data from Elliott Brown's initial characterization of Bt spores (top) original signature and (bottom) normalized. A signature centered around 900 GHz just below the water vapor line at 980 GHz was observed.	15
Figure 2. Data presented by Elliott Brown and his group to the Army Program Manager at the end of July 2012.	16
Appendix 3. Tables and figures for Achievement #3: Title: - MOLECULAR BEACON DESIGN.....	17
Table 1. Target DNA sequences for <i>hlyA</i> , <i>invA</i> and <i>Bt cry1Ac</i> genes.	17
Figure 1. Hairpin structure of MBs in quenched form. MBs designed for the detection of the <i>hlyA</i> gene of <i>Listeria monocytogenes</i> (A,B), the <i>invA</i> gene of <i>Salmonella Typhimurium</i> (C) and the transgene, <i>Bt cry1Ac</i> (D).	17
Appendix 4. Tables and figures for Achievement #4: Title: - MOLECULAR BEACON PRODUCTION.....	18
Figure 1. Hairpin structure of MBs in quenched form. MBs designed for the detection of the <i>hlyA</i> gene of <i>Listeria monocytogenes</i> (A), the <i>invA</i> gene of <i>Salmonella Typhimurium</i> (B), and the <i>Bt cry1Ac</i> gene of <i>Bacillus thuringiensis</i> (C).	18
Figure 2. Fluorescence observed in MB only (A) in quenched form (B) and MB in the presence of target DNA (GCT CAG ACA TGC CAC GGT ACA ACG (C) in unquenched form (D) under 302 nm UV light. The MB (5' - /5AmMC12/CCC CCG TTG TAC CGT GGC ATG TCT GAG CGG GG/3BHQ_2/ - 3') was designed to	

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

detect a target sequence (<i>invA</i>) found in <i>Salmonella</i> Typhimurium DT104. Final beacon concentration was 10 nM while final target concentration was 1 mM.	19
Appendix 5. Tables and figures for Achievement #5: Title: - USE OF MOLECULAR BEACONS FOR DETECTING FOODBORNE PATHOGENS, SALMONELLA TYPHIMURIUM AND LISTERIA MONOCYTOGENES AND INSECT PATHOGEN, BACILLUS THURINGIENSIS OR THE TRANSGENE, BT CRY1AC.	20
Figure 1. Fluorescence of 35 nM and 17.5 nM of molecular beacon (L3) designed for detecting <i>Listeria monocytogenes hlyA</i> gene in the presence of two concentrations (467 and 234 ng) of target genomic DNA within a buffer system using excitation wavelength of 485/20 nm and emission wavelength of 528/20 nm with sensitivity at 100. Error bars report standard error and letters above the bars indicate significant differences ($p < 0.05$).	20
Figure 2. Fluorescence of 35 nM and 17.5 nM of molecular beacon (L3) designed for detecting <i>Listeria monocytogenes hlyA</i> gene in the presence of two concentrations (467 and 234 ng) of target genomic DNA within an apple juice system using excitation wavelength of 485/20 nm and emission wavelength of 528/20 nm with sensitivity at 100. Error bars report standard error and letters above the bars indicate significant differences ($p < 0.05$).	21
Figure 3. Fluorescence observed in MB only in water (A) and 2% milk (B) in quenched form (C) and MB in the presence of target DNA in water (D) and 2% milk (E) in unquenched form (F) under 302 nm UV light. The MB was designed to detect a target sequence (<i>hlyA</i>) found in <i>Listeria monocytogenes</i> . Final beacon concentration was 1.17 nM (water) and 3.5 nM (2% milk), while final DNA concentration was 200 ng.	22
Figure 4. Fluorescence of molecular beacon (MB) designed to detect <i>Salmonella</i> Typhimurium <i>invA</i> gene alone and in two concentrations (400 and 800 ng) of target genomic DNA (A) and fluorescence of MB designed to detect <i>Listeria monocytogenes hlyA</i> gene alone and in three concentrations (50 ng, 100 ng and 200 ng) of target genomic DNA (B) within water and milk using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Error bars report standard error. The final concentration of MB higher concentration for water, water diluted, milk and milk diluted was 17.5 nM, 12.5 nM, 12.2 nM, and 8.6 nM, respectively, and the final concentration of MB lower concentration for water, water diluted, milk, and milk diluted was 8.75 nM, 6.25 nM, 6.1 nM and 4.3 nM, respectively. Treatments termed 'diluted' were diluted using milk or water to decrease the final concentration of MB, decreasing background fluorescence. Samples still contained the same quantity of genomic DNA.	23
Figure 5. Fluorescence of 8 nM of MB (<i>Bt cryIAC</i>) designed for detecting the <i>Bt cryIAC</i> gene in the presence of bacterial (<i>Bacillus thuringiensis</i>) genomic DNA (500 ng) in chicken broth (33%) using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 75. Error bars report standard error.	24
Figure 6. Fluorescence of 8 nM of MB (<i>Bt cryIAC</i>) designed for detecting the <i>Bt cryIAC</i> gene in the presence of bacterial (<i>Bacillus thuringiensis</i>) genomic DNA (500 ng) in chicken broth (100%) using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 75. Error bars report standard error.	25
Figure 7. Arabidopsis as a model system for detecting <i>Bacillus thuringiensis</i> . Fluorescence of 40 nM of molecular beacon (<i>Bt cryIAC</i>) designed for detecting <i>Bt cryIAC</i> gene in the presence of bacterial (<i>B. thuringiensis</i>) genomic DNA co-infiltrated into Arabidopsis plants observed in MB only, MB in the presence of 200 ng or 400 ng bacterial genomic DNA, 200 ng or 400 ng bacterial genomic DNA alone and water only under 302 nm UV light (above) and white light (below).	26
Figure 8. Fluorescence of 3.3 nM of MB (<i>Bt cryIAC</i>) designed for detecting the <i>Bt cryIAC</i> gene in the presence of bacterial (<i>Bacillus thuringiensis</i>) genomic DNA (using GenElute™ Bacterial Genomic DNA kit (SigmaAldrich, St. Louis, MO) following manufacturer's instructions) (diluted 10^0 to 10^{-8}) using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Genomic DNA concentrations were determined to be 125 ng/μl to 5 ng/μl from 8.54 log CFU/ml to 0.54 log CFU/ml. The limit of detection was determined to be 5.54 log CFU/ml, based on observed fluorescence. Error bars report standard error.	27
Figure 9. Fluorescence of 3.3 nM of MB (<i>Bt cryIAC</i>) designed for detecting the <i>Bt cryIAC</i> gene in the presence of bacterial (<i>Bacillus thuringiensis</i>) genomic DNA (using the Goldenberger et al. (1995) protocol) (diluted 10^0 to 10^{-2}) using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Genomic DNA concentrations were determined to be 1621 ng/μl to 17 ng/μl from 8.54 log CFU/ml to 6.54 log CFU/ml. The limit of detection was determined to be 5.54 log CFU/ml (the lowest log CFU/ml tested). Error bars report standard error.	28

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

Figure 10. Fluorescence of 33 nM of MB (*Bt cryIAc*) designed for detecting the *Bt cryIAc* gene in the presence of plant (transgenic tobacco) genomic DNA (740, 3700 or 7400 ng) in water using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Error bars report standard error....29

Figure 11. Fluorescence of 3.5 nM of MB (*Bt cryIAc*) designed for detecting the *Bt cryIAc* gene in the presence of plant (transgenic or non-transgenic tobacco) genomic DNA (4000 or 8000 ng) treated (heating at 95 °C for 5 min, vortex for 15 sec, or both (heating at 95 °C then vortexing for 15 sec)) using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100.....30

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

Overall Project Goals

Provide biological samples to collaborators for THz spectroscopy and to design, engineer and test molecular beacons with quantum dots as the fluorophore for the detection of foodborne pathogens and transgenes in intact plants and food systems in real time.

Research Goals

- (1) Design and provide collaborators with short single- (ssDNA) and double-DNA (dsDNA) sequences for THz spectroscopy.
- (2) Provide collaborators with crude and purified spores and vegetative cells from *Bacillus* species for detection using THz spectroscopy.
- (3) Design molecular beacons for the detection of gram-negative and gram-positive bacteria, *Salmonella* Typhimurium and *Listeria monocytogenes* respectively, and for the detection of the transgene, *BtCryIAC*
- (4) Manufacture above designed beacons for use in buffer, food systems (water, milk, apple juice, and chicken broth), and in plants (*Arabidopsis*)
- (5) Test activity of molecular beacons in the presence of target DNA, genomic DNA derived from bacteria and plants and using live bacteria and plants

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

Achievements

Achievement #1: Title: - TERAHERTZ SPECTROSCOPIC SIGNATURE FROM SHORT ARTIFICIAL DNA MOLECULES WITH WELL CONTROLLED STRUCTURE

Description

In this work we studied spectroscopic features of short artificial DNA molecules having a well defined 25 and 50 base pair (bp) sequence. Single-stranded (ssDNA) was synthesized, HPLC-purified, and further assessed using agarose gel electrophoresis at the University of Tennessee. Double-stranded (dsDNA) of known quantity and quality was prepared by annealing the complementary ssDNA strands. Samples of ssDNA and dsDNA were prepared with a final quantity of 4 mg each. Each sample was resuspended in 800 μ l of buffer solution consisting of 100 mM potassium acetate and 30 mM HEPES with pH 7.5 and stored at 4° C before measurements. The final DNA concentration of each sample was 5 μ g/ μ l.

Results

A total of six samples were produced: 25 bp ssDNA, complimentary strand 25 bp ssDNA, 25 bp dsDNA, 50 bp ssDNA, complimentary strand 50 bp ssDNA, 50 bp dsDNA. All samples were sent to collaborators at the final quantity of 2 mg/ μ l. Additionally, buffer containing no DNA was sent to serve as a negative control. DNA sequences are listed in Appendix 1, Table 1.

Presentations

Globus, T., B. Gelmont, C. N. Stewart, Jr., R. J. Millwood, and T. Khromova. 2010. THz spectroscopic signatures from short artificial DNA molecules, Oral presentation. ISSSR 2010, Springfield, MO. The 2010 International Symposium for Spectral Sensing Research (ISSSR). Abstract 101.

Achievement #2: Title: - TERAHERTZ SPECTROSCOPIC SIGNATURE FROM BACILLUS THURINGIENSIS AND BACILLUS SUBTILIS

Description

In this work, we studied spectroscopic signatures from *Bacillus* species. We provided collaborators with biological samples (purified spores and vegetative cells from *Bacillus subtilis* and *Bacillus thuringiensis* and sub-fractions (DNA, RNA, protein and extracellular material) as requested for use in THz spectroscopy.

Culture Preparation

Stock cultures of *Bacillus thuringiensis* subsp. *kurstaki* strains HD73-20 and HD73 obtained from the Department of Entomology and Plant Pathology at the University of Tennessee, Knoxville, were used. For vegetative cell production, cultures were grown individually overnight in 50 ml tryptic soy broth (TSB; Difco, Sparks, MD) at 30 °C, 150 rpm. For spore production, cultures were grown individually for 3 d in 50ml 1/3 TSB (10 g/L; Difco) at 30 °C, 150 rpm.

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

Vegetative and Spore Production and Purification

Vegetative and spore cultures were centrifuged at 7,000 rpm, 30 min to pellet. Vegetative pellets were resuspended in 8 ml sterile deionized water and divided into 2 ml microfuge tubes. Cells were centrifuged at 10,000 rpm, 15 min, supernatant removed and discarded, and pellets were stored at 4 °C until fractionation (less than 3 d).

Spore pellets were resuspended in 12 ml sterile 1M NaCl + 0.1% Triton X-100 and divided into 2 ml microfuge tubes. Tubes were vortexed, sonicated for 3 min, and centrifuged at 10,000 rpm, 15 min. This process was repeated three times, discarding supernatant each time. Pellets were then washed in sterile deionized water, vortexed, sonicated for 3 min, and centrifuged at 10,000 rpm, 15 min. Washing was repeated three times, discarding supernatant each time. Processed pellets were stored at 4 °C until fractionation (less than 3 d).

Cellular Fractionation

TRIzol® Reagent (Ambion, Life Technologies, Grand Island, NY) was used to fractionate purified vegetative cells and spores into extracellular debris/polysaccharides, RNA, DNA and protein according to manufacturer's instructions. Following homogenization with TRIzol® Reagent, the extracellular debris/polysaccharide fraction (lower red phenol-chloroform phase) was washed twice with 100% ethanol to remove any remnants of TRIzol® Reagent (phenol and guanidine isothiocyanate) and added chloroform for downstream spectroscopy. Extracellular debris/polysaccharides, DNA and protein fractions were stored at -20 °C and RNA fractions were stored at -80 °C.

Results

Dr. Elliott Brown's group reported a signature centered around 900 GHz just below the water vapor line at 980 GHz (Appendix 2, Figure 1). The sample handling and testing was done by Leamon Viveros, Ph.D. student of Elliott Brown.

Leamon Viveros traveled to Knoxville, TN mid-October 2011 to observe and prepare spore and vegetative samples of *Bacillus thuringiensis* and *Bacillus subtilis* for THz spectroscopy testing in Ohio. At the end of October 2011, Elliott Brown's group reported that the vegetative sample from *Bacillus* was scanned and two new peaks were observed. February 2012, fractionated spore and vegetative samples were shipped to Elliott Brown's group. These fractions included whole vegetative, vegetative RNA, vegetative DNA, vegetative protein, vegetative extracellular material, whole spore, spore RNA, spore DNA, spore protein, and spore extracellular material from two *Bacillus* species. Elliott Brown's group presented the data from August 2011 as part of their presentation to the DTRA THz Chem-Bio Workshop in Washington during the first week in April 2012 and as part of their presentation to the Army Program Manager at the end of July 2012 (Appendix 2, Figure 2). In December 2012, an additional six samples of vegetative and endospores derived from both strains of *Bacillus thuringiensis* were sent to Elliott Brown's group for additional THz spectroscopy measurements (Appendix 2, Table 1). In February 2013, six plates of confluent growth optimized for vegetative or endospore production derived from both strains of *Bacillus thuringiensis* were sent to Elliott Brown's group for additional THz spectroscopy measurements. Growth on agar media had proven easier to sample and process for *Rhodotorula* and was thus used for *Bacillus* samples.

Publications

Zhang, W., E. R. Brown, L. Viveros, K. P. Burris, and C. Neal Stewart, Jr. Submitted. Narrow terahertz attenuation signatures in *Bacillus thuringiensis*. *Journal of Biophotonics*.

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

Achievement #3: Title: - MOLECULAR BEACON DESIGN

Description

Molecular beacons are nucleic acid probes, consisting of between 15 and 25 nucleotides, with a fluorescent molecule at one end and a quencher molecule at the opposite end (Tyagi and Kramer, 1996). When unbound to their targets, molecular beacons have quenched fluorescence due to a hairpin structure that allows close proximity of the fluorophore to the quencher molecule. When in the presence of the target molecule, the molecular beacon binds to its complementary nucleic acid and fluorescence is triggered, allowing measurable detection. Molecular beacons have been successfully used in detecting the presence of foodborne pathogens *in vitro*, such as through the use of quantitative PCR. However, *in vivo* monitoring has only been demonstrated in mammalian cells (Bratu et al., 2003), not for detecting pathogens in foods or transgenes in intact plants. Therefore, we designed molecular beacons to specifically target *Listeria monocytogenes*, *Salmonella* and *Bacillus thuringiensis*.

Results

We designed molecular beacon probes for detecting *hlyA* and *invA* genes from *Listeria monocytogenes* (Gram-positive) and *Salmonella* spp. (Gram-negative) respectively and for the transgene, *Bt cry1Ac* (providing insecticide resistance and as a *Bacillus* model) (Appendix 3, Table 1). Several beacons were designed for each with the optimal confirmations chosen for production (Appendix 3, Figure 1).

References

- Bratu, D.P. et al. (2003) Visualization the distribution and transport of mRNAs in living cells. *Proc. Natl. Acad. Sci. USA* 100, 13308-13313.
- Tyagi, S. and Kramer, F.R. (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.* 14, 303-308.

Achievement #4: Title: - MOLECULAR BEACON PRODUCTION

Description

This work involved collaboration with Dr. Michael Stroschio and his group from the University of Illinois, Chicago. Dr. Stroschio's laboratory manufactured the molecular beacons with quantum dots designed in Achievement #3 for our use in detecting bacterial molecular signatures, specifically designed for the genes, *hlyA*, *invA*, and *Btcry1Ac* for the detection of *Listeria monocytogenes*, *Salmonella* and *Bacillus thuringiensis*, respectively (Appendix 4, Figure 1). Molecular beacon probes were manufactured by Dr. Stroschio's laboratory and provided to us in 10 μ M (*Listeria* and *Salmonella* MBs) and 1 μ M (*BtCry1Ac* MB) concentrations. Briefly, nucleic acids used to detect *Salmonella*, *L. monocytogenes*, and *B. thuringiensis* were obtained from Integrated DNA technology Inc. Each oligonucleotide was modified with an amide group with 12 extra carbon atoms on the 5' end, and a Black Hole Quencher II on the 3' end. EDC or EDAC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride) (Pierce) served as the crosslinker to activate the carboxyl groups on the quantum dot (QD; 10 mM e Fluor™ 605NC, eBioscience) surface to bind with the amino groups on DNA 5' terminal. These QDs are excited by 350-500 nm light and emit light with a wavelength of 605 nm.

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

Results

Prior to shipment, Dr. Stroschio and his laboratory confirmed beacon activities by testing for fluorescence against DNA of the target sequence. The molecular beacon designed for detecting the *invA* gene of *Salmonella* Typhimurium DT104, was successful in demonstrating increased fluorescence in the presence of its complementary target sequence (Appendix 4, Figure 2)

Achievement #5: Title: - USE OF MOLECULAR BEACONS FOR DETECTING FOODBORNE PATHOGENS, SALMONELLA TYPHIMURIUM AND LISTERIA MONOCYTOGENES AND INSECT PATHOGEN, BACILLUS THURINGIENSIS OR THE TRANSGENE, BT CRY1AC

Description

Recent and recurring outbreaks in our food supply and post-September 11 threats of bioterrorism have prompted the need for better detection methodologies for bacterial pathogens and toxins. Persons are at risk for the harmful effects caused by many foodborne pathogenic bacteria and their toxins. Foodborne illnesses are a continuous threat to public and military personnel health and Scallan et al. (2011) estimate that the 31 major foodborne pathogens account for nearly 9.4 million people becoming sick, more than 55,961 hospitalizations, and 1,351 deaths from foodborne illness each year in the United States with an estimated economic cost of \$152 billion annually (Scharff, 2010). Standard methods exist for quantifying/detecting microorganisms in foods; however, these methods are relatively limited in specificity, sensitivity, and time. The benefits of creating and utilizing a technology that can detect bacterial pathogens in real-time and on-the-food are the reduction and/or elimination of disease and mortality caused by foodborne bacteria. This advancement will aid in the detection of bacterial pathogens at all steps of the food supply chain, preventing both disease and mortality and reducing economic costs.

We developed molecular beacon-based technology for detecting entire organisms or populations of intact organisms for specific signature molecules *in vivo* that were able to detect and monitor low quantities of pathogen targets in real-time, in efforts to detect and monitor for foodborne bacterial pathogens within food and crop systems. We designed target DNA sequences for detecting *hlyA* and *invA* genes from *Listeria monocytogenes* and *Salmonella* sp. respectively and for the transgene, *Bt cry1AC*, and have prepared molecular beacons for specific targets for use in real-time monitoring. We demonstrated the ability to detect the presence of target sequence and nucleic acid extracted from bacteria (*in vitro* monitoring) in several liquid food systems and within plant tissue, which demonstrates use in monitoring for bacterial pathogens in foods and crops. We successfully detected changes in fluorescence at concentrations from ca. 1.2 to 40 nM, depending upon respective target and target concentration.

Culture preparation and bacterial genomic DNA extraction

Stock cultures of *Salmonella* Typhimurium DT104 strain 2576 and *L. monocytogenes* strain Scott A, obtained from the Department of Food Science and Technology at the University of Tennessee, Knoxville, and *B. thuringiensis* subsp. *kurstaki* strain HD73 obtained from the Department of Entomology and Plant Pathology at the University of Tennessee, Knoxville, were used. Cultures were grown individually overnight in tryptic soy broth (TSB; Difco, Sparks, MD) at 30-35 °C (depending upon bacteria), 150 rpm. Genomic DNA was extracted using the GenElute™ Bacterial Genomic DNA kit (SigmaAldrich, St. Louis, MO) following manufacturer's instructions and quantified using a NanoDrop (ND-1000, Thermo Scientific, Wilmington, DE).

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

Plant genomic DNA extraction

We have grown 3 lines in duplicate of transgenic tobacco plants, containing the *Bt cryIAC* gene for insecticidal resistance, and one control line for tissue extraction and molecular beacon infiltration. Plant genomic DNA was extracted using a CTAB method. Briefly, 2 g plant material was ground in liquid nitrogen and extracted with DNA extraction buffer [2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 0.2% b-mercaptoethanol] at 65 °C for 15 min. An equal volume of phenol-chloroform/isoamylalcohol was added, centrifuged for 15 min, 13,000 rpm at room temperature and aqueous phase obtained. Chloroform/isoamylalcohol (24:1) was added to this aqueous phase and centrifuged for 15 min, 13,000 rpm at room temperature and repeated. The aqueous phase was precipitated with 0.7 volumes of isopropanol at -80 °C overnight. Pelleted DNA was washed with 70% ethanol, air dried and resuspended in sterile water. DNA was stored at -20 °C prior to use. Genomic DNA from bacteria and plants was quantified using a NanoDrop (ND-1000, Thermo Scientific, Wilmington, DE).

Fluorescence detection

Luminescent semiconductor quantum-dot—based molecular beacons were tested in the presence of their genomic DNA in multiple systems, RNase/DNase free water, 2% milk, apple juice, chicken broth and/or co-infiltrated into 6 wk old *Arabidopsis thaliana* ‘Columbia’ leaves. Fluorescence in presence of genomic DNA in water, 2% milk, apple juice or chicken broth was measured using a Synergy HT microplate reader with excitation of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Fluorescence observed within the plant tissue or in microfuge tubes was observed visually under UV light (302 nm) (LKB 2011 Macrovue transilluminator, Bromma, Sweden).

MBs designed to detect the *S. Typhimurium invA* gene were measured for fluorescence alone and in the presence of two concentrations (400 and 800 ng) of target genomic DNA in water and milk. MBs designed to detect *L. monocytogenes hlyA* gene were measured for fluorescence alone and in the presence of three concentrations (50 ng, 100 ng, and 200 ng) of target genomic DNA in water and milk. MBs were tested at a high concentration (MB higher concentration) and at a low concentration (MB lower concentration). The final concentration of MB higher concentration for water, water diluted, milk and milk diluted was 17.5 nM, 12.5 nM, 12.2 nM, and 8.6 nM, respectively, and the final concentration of MB lower concentration for water, water diluted, milk, and milk diluted was 8.75 nM, 6.25 nM, 6.1 nM and 4.3 nM, respectively. Treatments termed ‘diluted’ were diluted using water or milk to decrease the final concentration of MB, decreasing background fluorescence. Samples still contained the same quantity of genomic DNA.

Results

Each quantum-dot-based MB probe was tested for enhanced fluorescence in water, buffer, milk, apple juice, chicken broth, and/or plant tissue when in the presence of its respective signature molecule derived from *Listeria monocytogenes*, *Bacillus thuringiensis*, and *Salmonella Typhimurium*.

The molecular beacon probe designed for detecting *Listeria monocytogenes* was tested in both buffer (Appendix 5, Figure 1) and apple juice systems (Appendix 5, Figure 2). Fluorescence was monitored for two concentrations of *Listeria* beacon (17.5 nM and 35 nM) in the presence of two concentrations of target genomic DNA (234 ng and 437 ng). Fluorescence was significantly higher for both concentrations of beacon and target genomic DNA when compared to their respective controls (Appendix 5, Figure 1). Fluorescence did not differ between the two concentrations of beacon tested; however, the higher the

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

concentration of target genomic DNA, the greater the fluorescence for both concentrations of beacon (Appendix 5, Figure 1). We also found that fluorescence for this beacon (17.5 nM) in the presence of 234 ng of target genomic DNA was significantly greater than the control in 9% apple juice adjusted to pH 5.5 (Appendix 5, Figure 2). However, it appears that the beacons are susceptible to false negatives possibly due to low pH.

As illustrated in Appendix 5, Figure 3, fluorescence could be visualized with the naked eye using UV 302 nm when the genomic DNA concentration was 200 ng when either water or milk was used as the substrate. When a lower concentration of MB probe was used, less background fluorescence was observed (Appendix 5, Figure 4).

Higher fluorescence was observed for MBs designed to detect *S. Typhimurium invA* gene when in the presence of two concentrations (400 and 800 ng) of bacterial genomic DNA than in MB alone. When water was used as the substrate, equal increases in fluorescence above MB alone were observed for both 400 and 800 ng of bacterial genomic DNA (Appendix 5, Figure 4A). However, when milk was used as the substrate, the increase in fluorescence above MB alone was greater for 800 ng of bacterial genomic DNA than 400 ng genomic DNA, indicating a higher amount of signature molecule is necessary for detection in food substrates such as milk (Appendix 5, Figure 4A).

Similar results were observed for MBs designed to detect *L. monocytogenes hlyA* gene when in the presence of three concentrations (50 ng, 100 ng and 200 ng) of bacterial genomic DNA (Appendix 5, Figure 4B). When water was used as the substrate, equal increases in fluorescence above MB alone were observed for 50, 100 and 200 ng of bacterial genomic DNA (Appendix 5, Figure 4B). When milk was used as the substrate, equal increases in fluorescence above MB alone (higher concentration) were observed for 50, 100 and 200 ng of bacterial genomic DNA; however, when a lower concentration of MB was used, equal increases in fluorescence above MB alone were observed for 100 and 200 ng of bacterial genomic DNA, indicating a higher concentration of bacterial genomic DNA and MB probe are necessary for detection in a food substrate (Appendix 5, Figure 4B).

We also tested the ability of the *Bt cryIAC* and *Salmonella* beacons to detect target bacterial genomic DNA in chicken broth. Increased fluorescence was observed when the *Bt cryIAC* beacon (8 nM) was in the presence of 500 ng of bacterial genomic DNA in chicken broth (33%) (Appendix 5, Figure 5). However, when the *Bt cryIAC* beacon (8 nM) was tested in the presence of 500 ng bacterial genomic DNA in chicken broth (100%), no difference in fluorescence was observed between beacon alone and beacon in the presence of target bacterial DNA (Appendix 5, Figure 6), indicating potential limitations to use in complex food systems. No increase in fluorescence was observed when the *Salmonella* beacon (13 nM) was tested in the presence of 800 ng bacterial genomic DNA in chicken broth (33%) (data not shown).

Since it was possible to observe increases in fluorescence above MB probe alone in liquid substrates (water and milk), we wanted to determine the ability to detect the presence of signature molecules within a more complex substrate, plant tissues (Appendix 5, Figure 7). As illustrated in Appendix 5, Figure 7, fluorescence could be visualized with the naked eye using UV 302 nm when the *Bt cryIAC* beacon probe was co-infiltrated into Arabidopsis in the presence of bacterial genomic DNA (100 ng or 200 ng). An increase in fluorescence was observed above MB probe alone and no fluorescence was observed within the plant tissues for the MB probe alone, bacterial genomic DNA alone (100 ng or 200 ng) or water treatments (Appendix 5, Figure 7), indicating the potential use for *in planta* detection.

In efforts to identify the level of detection for specific signature molecules, we developed protocols for testing the detection of bacteria at varying growth levels (CFU/ml). We used two bacterial genomic DNA extraction techniques from overnight growth of *Bacillus thuringiensis* cultures (diluted 10^0 to 10^{-8}) for

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

MB exposure. Bacterial genomic DNA was extracted using either the GenElute™ Bacterial Genomic DNA kit (SigmaAldrich, St. Louis, MO) following manufacturer's instructions (diluted 10^0 to 10^{-8}) or using a crude extraction protocol modified from Goldenberger et al. (1995) with Triton X-100 buffer and heating (diluted 10^0 to 10^{-2}).

Genomic DNA concentrations obtained from extractions of *B. thuringiensis* subsp. *kurstaki* strain HD73 were determined to be 125 ng/μl to 5 ng/μl from 8.54 log CFU/ml to 0.54 log CFU/ml respectively using the GenElute™ Bacterial Genomic DNA kit and 1621 ng/μl to 17 ng/μl from 8.54 log CFU/ml to 6.54 log CFU/ml using the protocol from Goldenberger et al. (1995). We found that using GenElute™ Bacterial genomic DNA extracts, the level of detection was approximately 5.54 log CFU/ml (Appendix 5, Figure 8). When genomic DNA extracts from the more crude Goldenberger et al. (1995) protocol, it was observed that the buffer (no bacterial genomic DNA) contributed to the observed increased fluorescence (Appendix 5, Figure 9).

We obtained approximately 150-300 μg plant genomic DNA, depending on tobacco plant line, when extracted using the CTAB method. The *Bt cryIAC* beacon (33 nM) was tested in the presence of 740, 3700 or 7400 ng plant genomic DNA. Only a slight increase in fluorescence was observed in the MB when in the presence of 7400 ng plant genomic DNA (Appendix 5, Figure 10). Similar results were observed when we treated the plant genomic DNA by both heating to 95 °C for 5 min and then vortexing for 15 (Appendix 5, Figure 11). However, this assay needs to be repeated to determine if the differences are significant.

Presentations

Burris, K.P., T-C. Wu, M. Strosio, M. Vasudev, R. Millwood, and C. N. Stewart, Jr. 2011. Rapid detection and monitoring of bacterial pathogens and transgenes using molecular beacon technology. Nanoelectronic Devices for Defense & Security Conference (August 29-September 1, 2011), Brooklyn, NY.

Burris, K.P., Reggie Millwood, and C. Neal Stewart, Jr. 2011. Biomolecular architectures molecular biology: Molecules for THz spectroscopy and molecular beacon technology. ARO-DTRA PIs project review meeting (July 13-14, 2011), Washington, DC. (invited).

Burris, K.P., M. Strosio, T.-C. Wu, M. Vasudev, R.J. Millwood and C. Neal Stewart, Jr. 2010. Real-time mega-nano detection of foodborne pathogens and transgenes using molecular beacon technology. International Symposium on Spectral Sensing Research (June 21-24 2010), Springfield, MO. Abstract 210. (invited).

Burris, K.P., M. Strosio, M. Vasudev, D. Wu, and C. Neal Stewart, Jr. 2009. In vivo mega-nano detection of foodborne pathogens and transgenes using molecular beacon technology. Nanoelectronic Devices for Defense & Security Conference (Sept 28-Oct 2, 2009), Fort Lauderdale, FL. (invited).

Vasudev, M., T.-C. Wu, M. Dutta, M.A. Strosio, S. Guthrie, M. Reed, K. Burris, and C. N. Stewart, Jr. 2009. Optoelectric signatures of DNA based hybrid nanostructures. Nanoelectronic Devices for Defense & Security Conference (Sept 28-Oct 2, 2009), Fort Lauderdale, FL.

Burris, K.P., and C. Neal Stewart, Jr. 2009. Mega-nano structure for detecting foodborne pathogens. ARO PIs project meeting (March 20, 2009), Raleigh NC. (invited).

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

Publications

Burris, K.P., T.-C. Wu, M. Vasudev, M.A. Strosio, R. J. Millwood, and C. Neal Stewart, Jr. Submitted. Mega-nano detection of signature molecules using molecular beacon technology and semiconductor quantum dot technologies. *IEEE Transactions on NanoBioscience*.

Burris, K.P. and C. Neal Stewart, Jr. 2012. Fluorescent nanoparticles: sensing pathogens and toxins in foods and crops. *Trends in Food Science & Technology* 28:143-152.

Vasudev, M., T.-C. Wu, M. Dutta, M.A. Strosio, S. Guthrie, M. Reed, **K.P. Burris**, and C. Neal Stewart, Jr. 2011. Optoelectronic signatures of DNA based hybrid nanostructures. *IEEE Transactions on Nanotechnology*. 10:35-43.

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Bratu, D.P. *et al.* (2003) Visualization the distribution and transport of mRNAs in living cells. *Proc. Natl. Acad. Sci. USA* 100, 13308-13313.

Goldenberger, D. *et al.* (1995) A simple “universal” DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification. *PCR Methods Applic* 4, 368–370.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A. Widdowson, and S. L. Roy. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg Infect Dis* 17:7-15.

Scharff, R. L. 2010. Health-related costs from foodborne illness in the United States. Washington, DC: The Produce Safety Project at Georgetown University.

**Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013**

***Appendix 1. Tables and figures for Achievement #1: Title: -
TERAHERTZ SPECTROSCOPIC SIGNATURE FROM SHORT
ARTIFICIAL DNA MOLECULES WITH WELL CONTROLLED
STRUCTURE.***

Table 1. Short synthetic 25 bp and 50 bp sequences.

Sample name	Sequence
ssDNA 25 -----	5' - TCG ATC GAT CGA TCG ATC GAT CGA T - 3'
ssDNA 25 complement---	3' - ATC GAT CGA TCG ATC GAT CGA TCG A - 5'
ssDNA 50-----	5' - TCG ATC GAT CGA TCG ATC GAT CGA TTC GAT CGA TCG ATC GAT CGA TCG AT - 3'
ssDNA 50 complement---	3' - ATC GAT CGA TCG ATC GAT CGA TCG AAT CGA TCG ATC GAT CGA TCG ATC GA - 5'
dsDNA 25 -----	5' - TCG ATC GAT CGA TCG ATC GAT CGA T - 3'
	3' - ATC GAT CGA TCG ATC GAT CGA TCG A - 5'
dsDNA 50 -----	5' - TCG ATC GAT CGA TCG ATC GAT CGA TTC GAT CGA TCG ATC GAT CGA TCG AT - 3'
	3' - ATC GAT CGA TCG ATC GAT CGA TCG AAT CGA TCG ATC GAT CGA TCG ATC GA- 5'

**Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013**

**Appendix 2. Tables and figures for Achievement #2: Title: -
TERAHERTZ SPECTROSCOPIC SIGNATURE FROM BACILLUS
THURINGIENSIS AND BACILLUS SUBTILIS.**

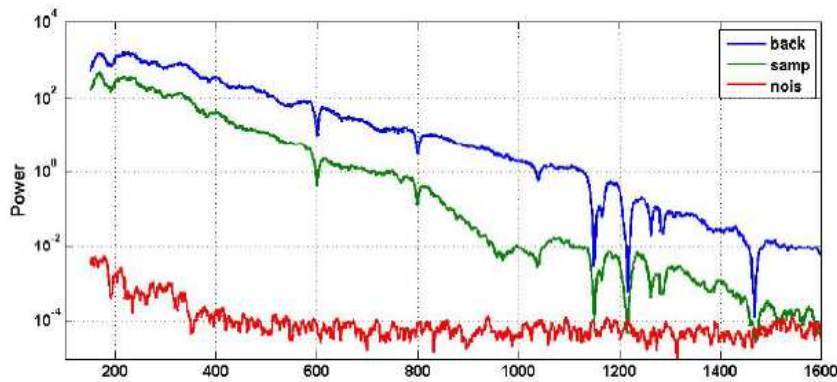
Table 1. Wet weights and enumeration of six samples of vegetative cells and endospores derived from the HD73-20 strain of *Bacillus thuringiensis* for THz spectroscopy measurements.

Sample	Log (CFU/ml)	Replicate	Tube weight (g)	Tube + wet pellet weight (g)	Pellet weight (g)	Amount of initial culture (ml)
<i>Bacillus thuringiensis</i> HD73-20 heated and grown in 1/3 TSB	8.03	A	13.62	14.20	0.58	48
<i>Bacillus thuringiensis</i> HD73-20 heated and grown in 1/3 TSB	8.01	B	13.62	14.23	0.61	48
<i>Bacillus thuringiensis</i> HD73-20 grown in 1/3 TSB	7.93	A	13.47	14.01	0.54	47
<i>Bacillus thuringiensis</i> HD73-20 grown in 1/3 TSB	8.00	B	13.54	14.06	0.52	47
<i>Bacillus thuringiensis</i> HD73-20 grown in TSB	8.79	A	13.62	14.08	0.46	48
<i>Bacillus thuringiensis</i> HD73-20 grown in TSB	8.75	B	13.41	13.91	0.50	48

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

Preliminary BT Transmission Results

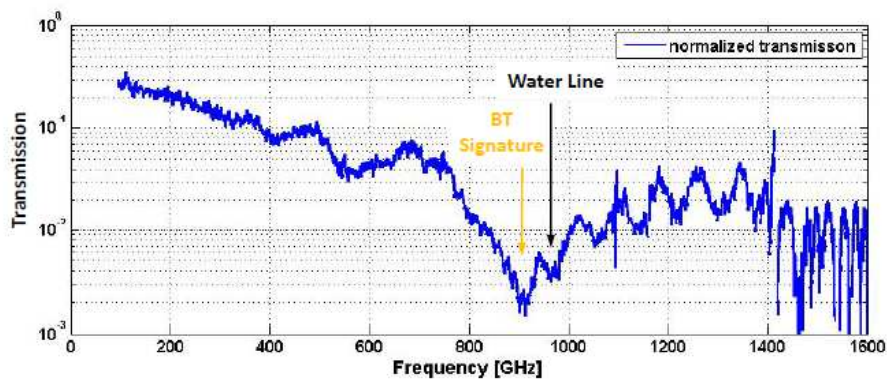
(embedded in filter paper)



L. Viveros and E.R. Brown
25 August 2011

Preliminary BT Transmission Results

(embedded in filter paper)



L. Viveros and E.R. Brown
25 August 2011

Figure 1. Data from Elliott Brown's initial characterization of Bt spores (top) original signature and (bottom) normalized. A signature centered around 900 GHz just below the water vapor line at 980 GHz was observed.

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

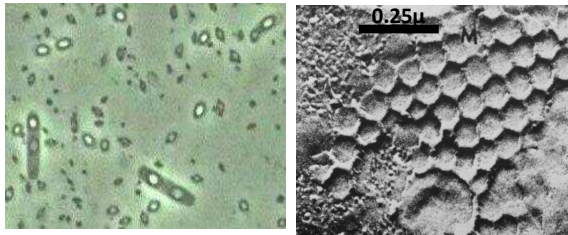


***Bacillus thuringiensis* (Bt) Characterization**



***Bacillus thuringiensis* Sample**

- Our largest absorption signature from any biosample
- Bacteria was sonicated & insoluble components separated for analysis (U Tenn, Knoxville)



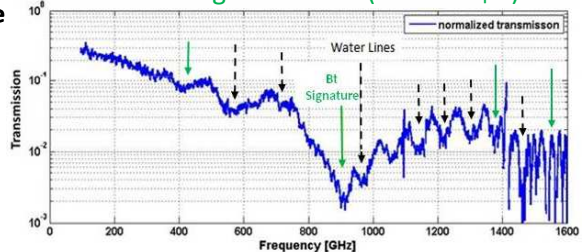
Accomplishments

- A dramatic & possibly specific signature for *B. cereus* family (*B. anthracis*, *B. cereus*, & *B. thuringiensis*)
- Much stronger and at lower frequency than signature from previously studied *B. subtilis* (at 1035 GHz)
- First known THz microsampling technique used (0.5 uL)
- Acquired fieldable THz spectrometer (Emcore PB7200) for mobile sampling in secure laboratories, and for operation in reflection mode.

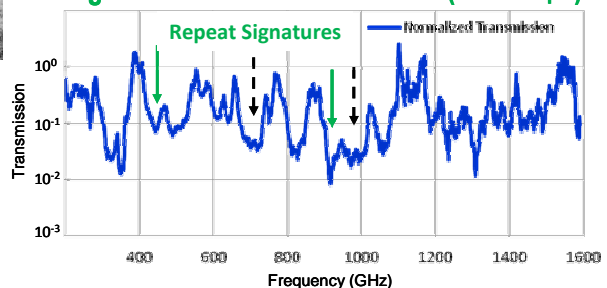


Experimental Results

Whole Vegetative Cell (0.5 mL Sample)



Vegetative Cell Insoluble Material (Microsample)



Conclusion

- Suggests that Bt THz signature(s) are from cell wall and membrane material, not endospores
- Justifies follow-up testing of *B. anthracis* and ricin at FBI laboratory.

Figure 2. Data presented by Elliott Brown and his group to the Army Program Manager at the end of July 2012.

**Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013**

**Appendix 3. Tables and figures for Achievement #3: Title: -
MOLECULAR BEACON DESIGN.**

Table 1. Target DNA sequences for *hlyA*, *invA* and *Bt cryIAC* genes.

Gene	Target DNA sequence
<i>hlyA</i>	CAT CGT CCA TCT ATT TGC CAG GTA
<i>hlyA</i>	GAT TT GAT CCG CGT GTT T
<i>invA</i>	GCT CAG ACA TGC CAC GGT ACA ACG
<i>BtCryIAC</i>	CTC TCA ATG GGA CGC CTT TCT TGT AC

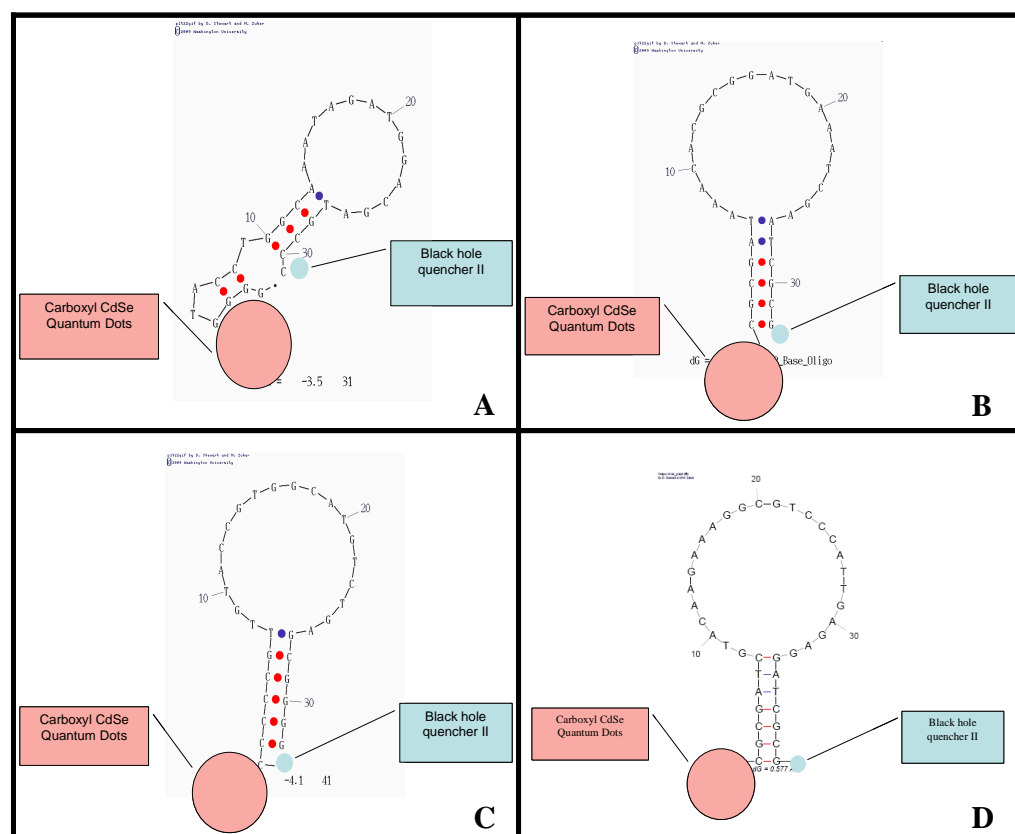


Figure 1. Hairpin structure of MBs in quenched form. MBs designed for the detection of the *hlyA* gene of *Listeria monocytogenes* (A,B), the *invA* gene of *Salmonella Typhimurium* (C) and the transgene, *Bt cryIAC* (D).

**Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013**

**Appendix 4. Tables and figures for Achievement #4: Title: -
MOLECULAR BEACON PRODUCTION.**

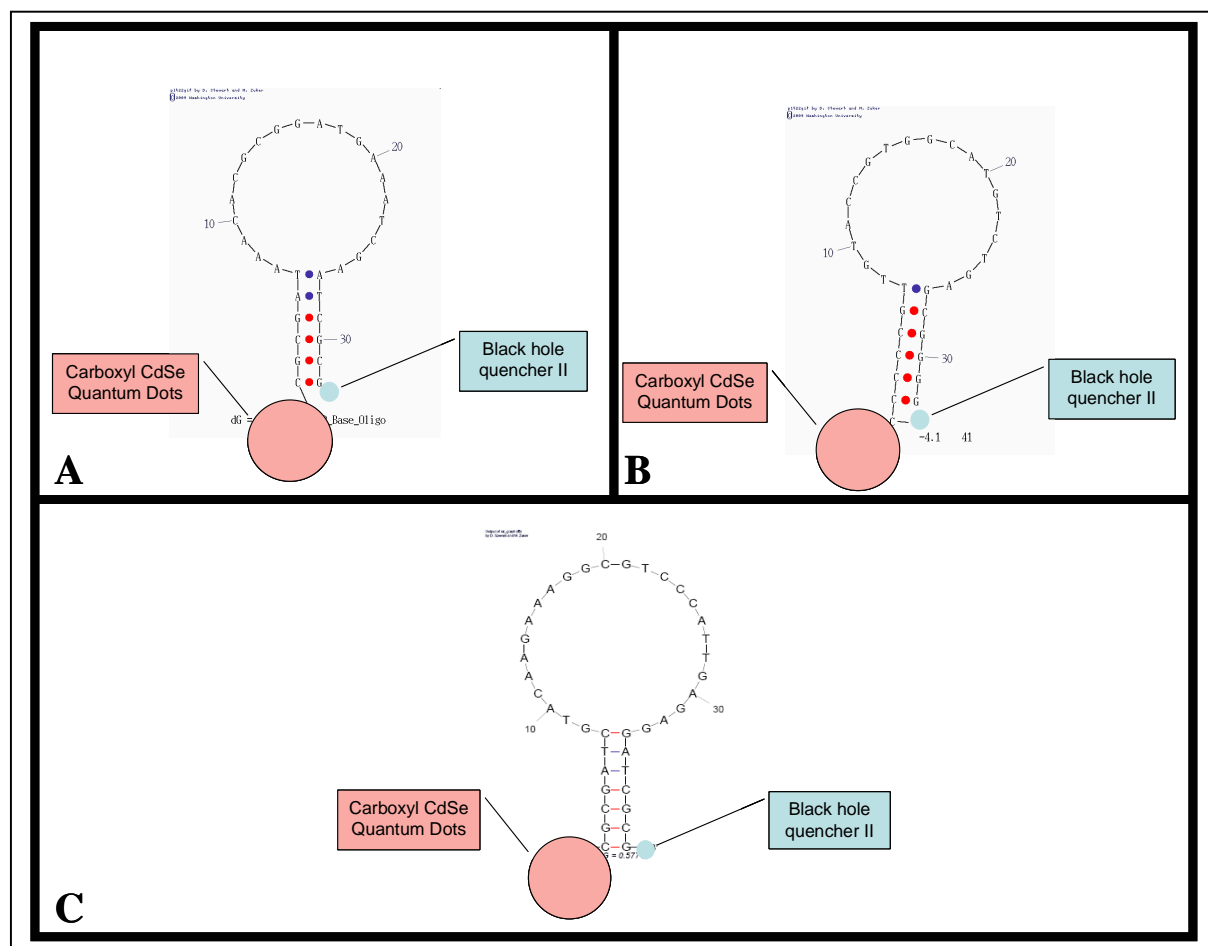


Figure 1. Hairpin structure of MBs in quenched form. MBs designed for the detection of the *hlyA* gene of *Listeria monocytogenes* (A), the *invA* gene of *Salmonella Typhimurium* (B), and the *Bt cryIAc* gene of *Bacillus thuringiensis* (C).

**Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013**

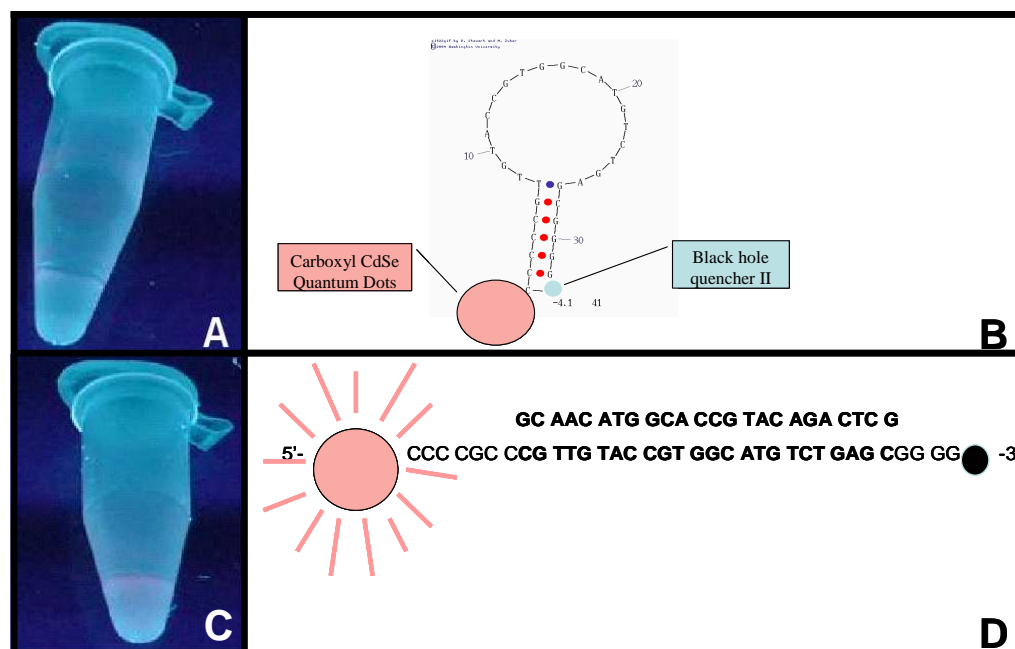


Figure 2. Fluorescence observed in MB only (A) in quenched form (B) and MB in the presence of target DNA (GCT CAG ACA TGC CAC GGT ACA ACG (C) in unquenched form (D) under 302 nm UV light. The MB (5' - /5AmMC12/CCC CCG TTG TAC CGT GGC ATG TCT GAG CCG GG/3BHQ_2/ - 3') was designed to detect a target sequence (*invA*) found in *Salmonella Typhimurium* DT104. Final beacon concentration was 10 nM while final target concentration was 1 mM.

Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013

Appendix 5. Tables and figures for Achievement #5: Title: - USE OF MOLECULAR BEACONS FOR DETECTING FOODBORNE PATHOGENS, SALMONELLA TYPHIMURIUM AND LISTERIA MONOCYTOGENES AND INSECT PATHOGEN, BACILLUS THURINGIENSIS OR THE TRANSGENE, BT CRY1AC.

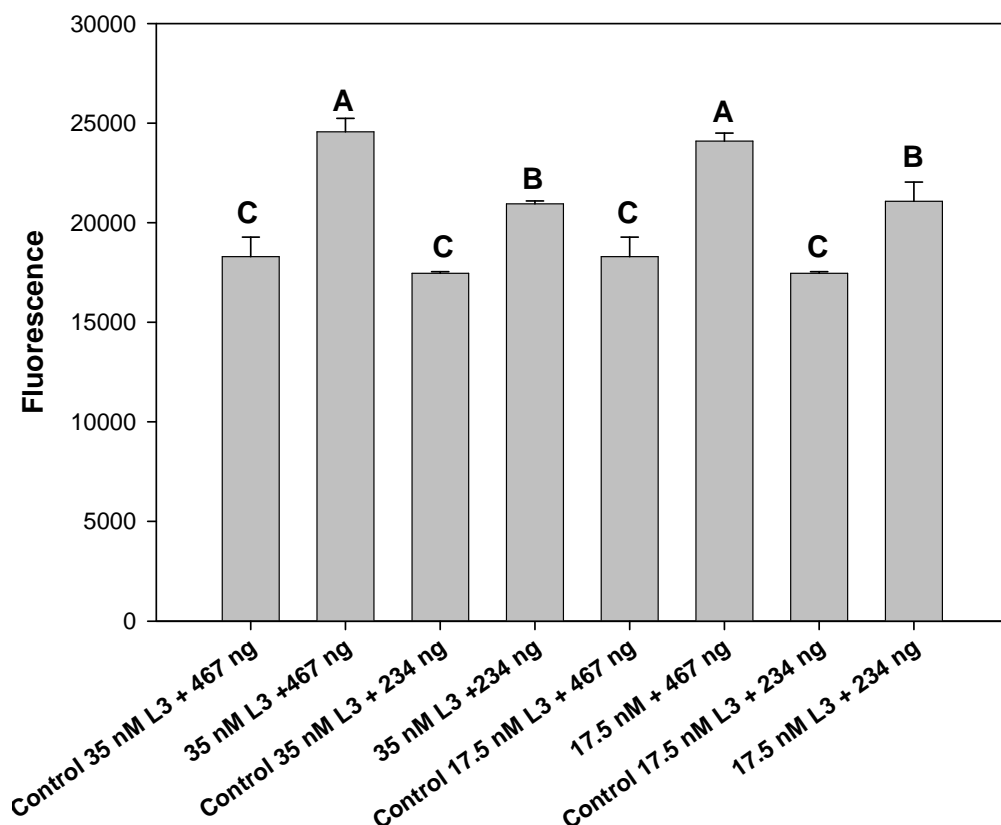


Figure 1. Fluorescence of 35 nM and 17.5 nM of molecular beacon (L3) designed for detecting *Listeria monocytogenes hlyA* gene in the presence of two concentrations (467 and 234 ng) of target genomic DNA within a buffer system using excitation wavelength of 485/20 nm and emission wavelength of 528/20 nm with sensitivity at 100. Error bars report standard error and letters above the bars indicate significant differences ($p<0.05$).

**Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013**

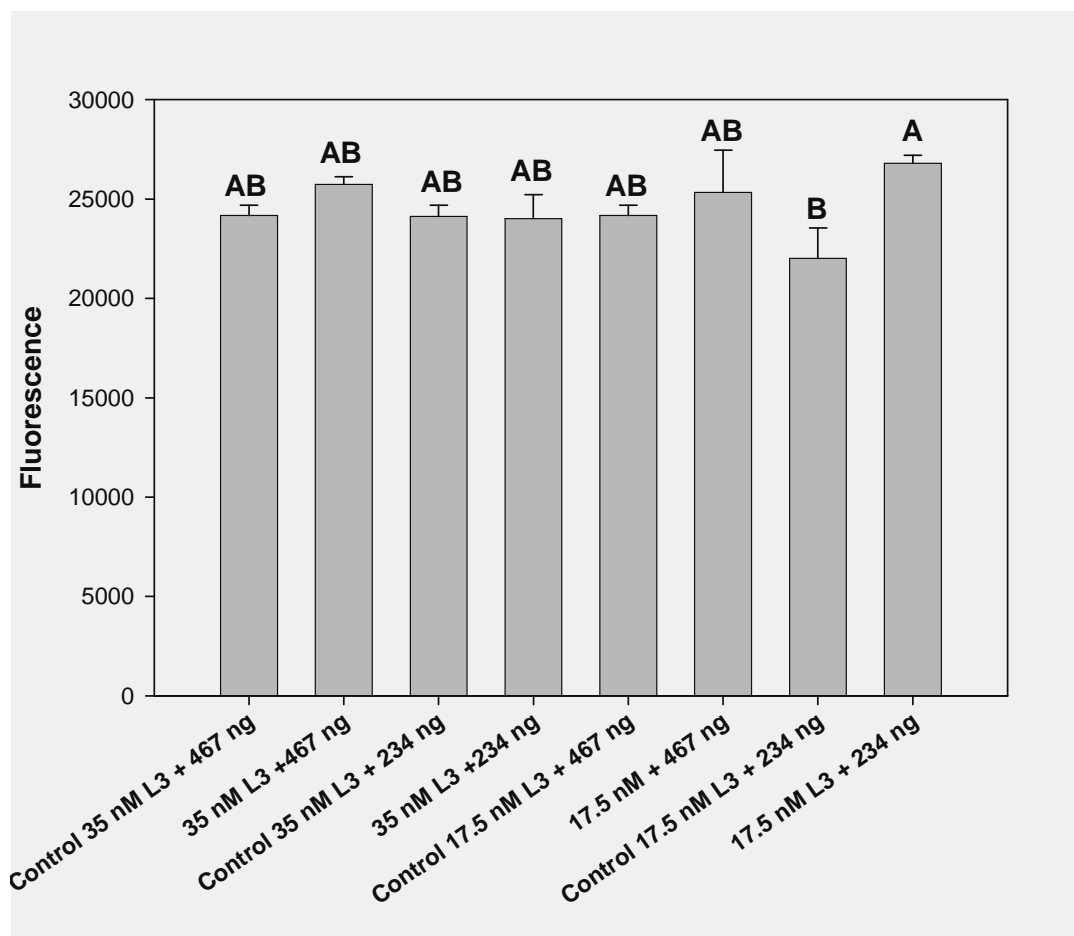


Figure 2. Fluorescence of 35 nM and 17.5 nM of molecular beacon (L3) designed for detecting *Listeria monocytogenes hlyA* gene in the presence of two concentrations (467 and 234 ng) of target genomic DNA within an apple juice system using excitation wavelength of 485/20 nm and emission wavelength of 528/20 nm with sensitivity at 100. Error bars report standard error and letters above the bars indicate significant differences ($p<0.05$).

**Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013**

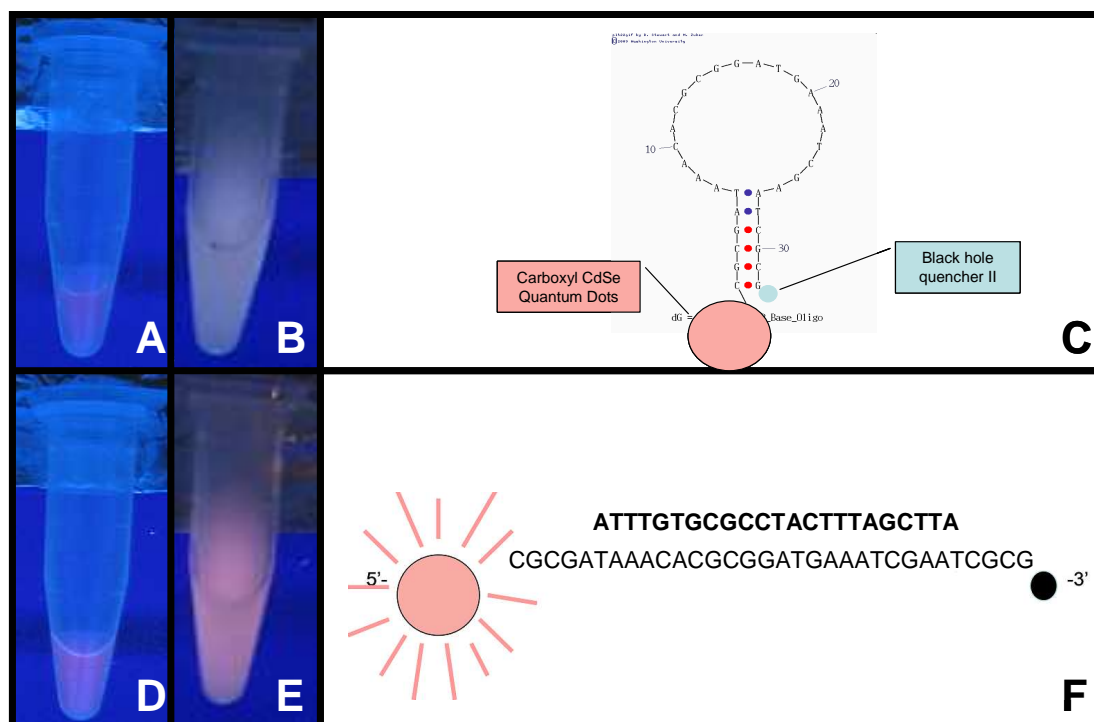


Figure 3. Fluorescence observed in MB only in water (A) and 2% milk (B) in quenched form (C) and MB in the presence of target DNA in water (D) and 2% milk (E) in unquenched form (F) under 302 nm UV light. The MB was designed to detect a target sequence (*hlyA*) found in *Listeria monocytogenes*. Final beacon concentration was 1.17 nM (water) and 3.5 nM (2% milk), while final DNA concentration was 200 ng.

Scientific Progress and Accomplishments for Report period May 1, 2008 through March 31, 2013

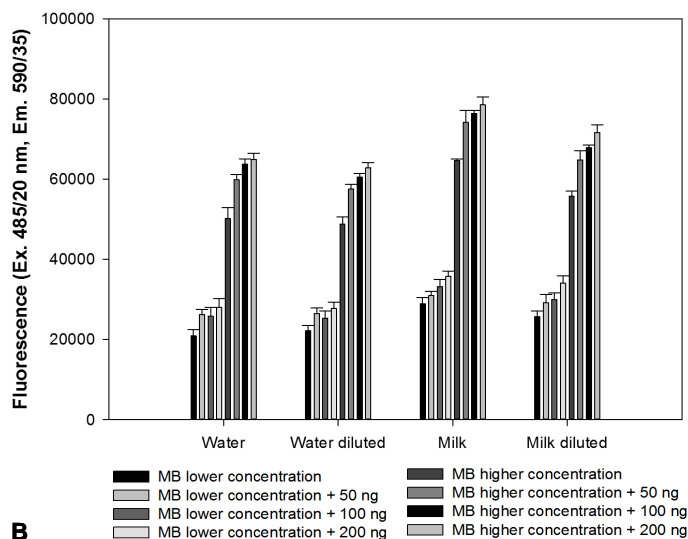
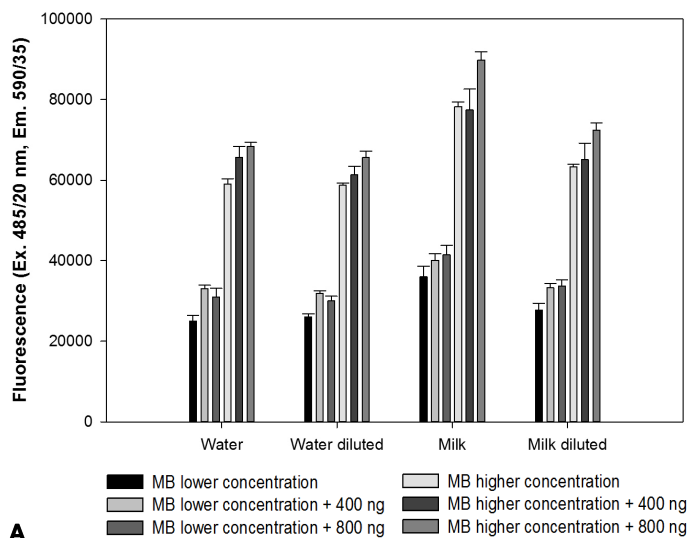


Figure 4. Fluorescence of molecular beacon (MB) designed to detect *Salmonella Typhimurium invA* gene alone and in two concentrations (400 and 800 ng) of target genomic DNA (A) and fluorescence of MB designed to detect *Listeria monocytogenes hlyA* gene alone and in three concentrations (50 ng, 100 ng and 200 ng) of target genomic DNA (B) within water and milk using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Error bars report standard error. The final concentration of MB higher concentration for water, water diluted, milk and milk diluted was 17.5 nM, 12.5 nM, 12.2 nM, and 8.6 nM, respectively, and the final concentration of MB lower concentration for water, water diluted, milk, and milk diluted was 8.75 nM, 6.25 nM, 6.1 nM and 4.3 nM, respectively. Treatments termed ‘diluted’ were diluted using milk or water to decrease the final concentration of MB, decreasing background fluorescence. Samples still contained the same quantity of genomic DNA.

Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013

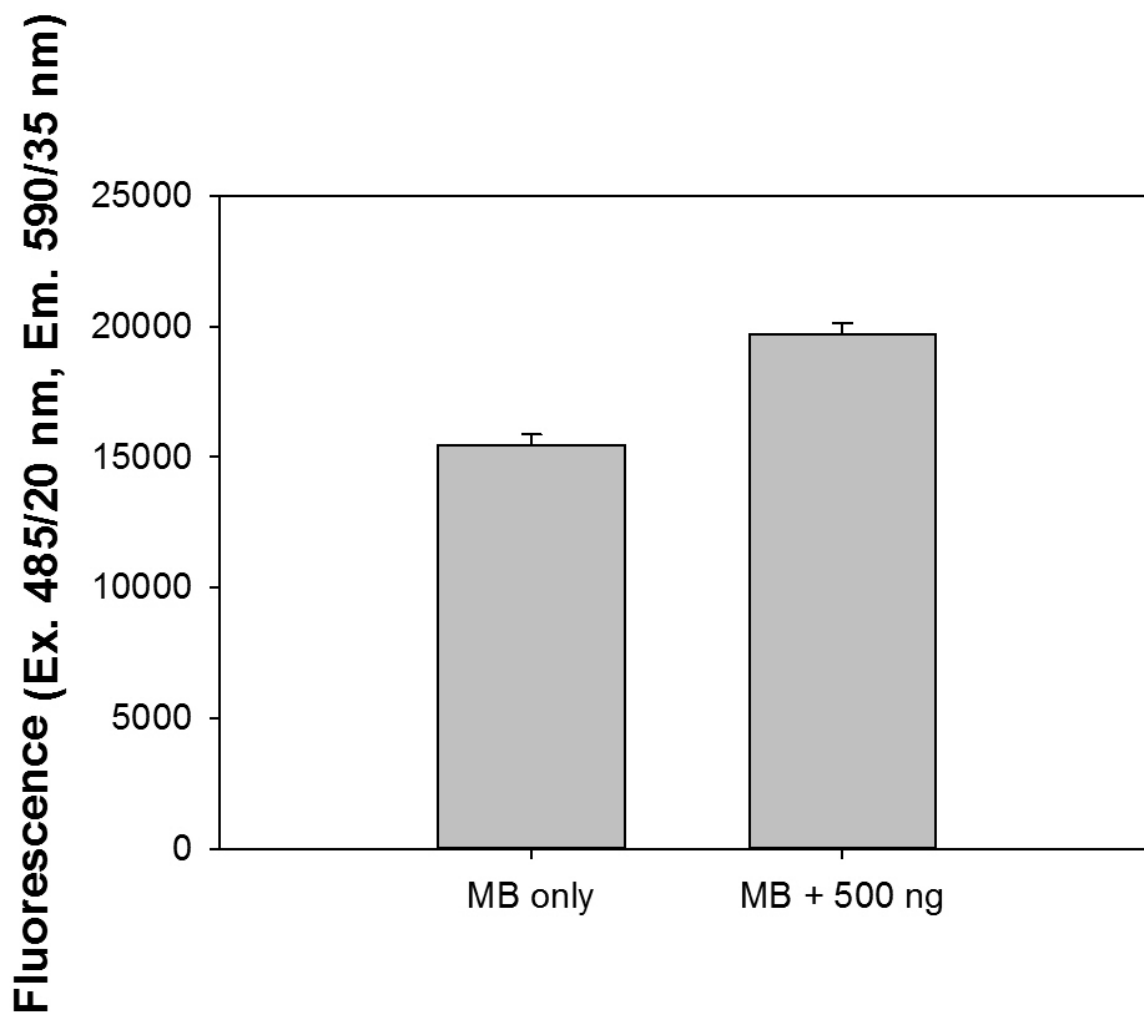


Figure 5. Fluorescence of 8 nM of MB (*Bt cryIAc*) designed for detecting the *Bt cryIAc* gene in the presence of bacterial (*Bacillus thuringiensis*) genomic DNA (500 ng) in chicken broth (33%) using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 75. Error bars report standard error.

Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013

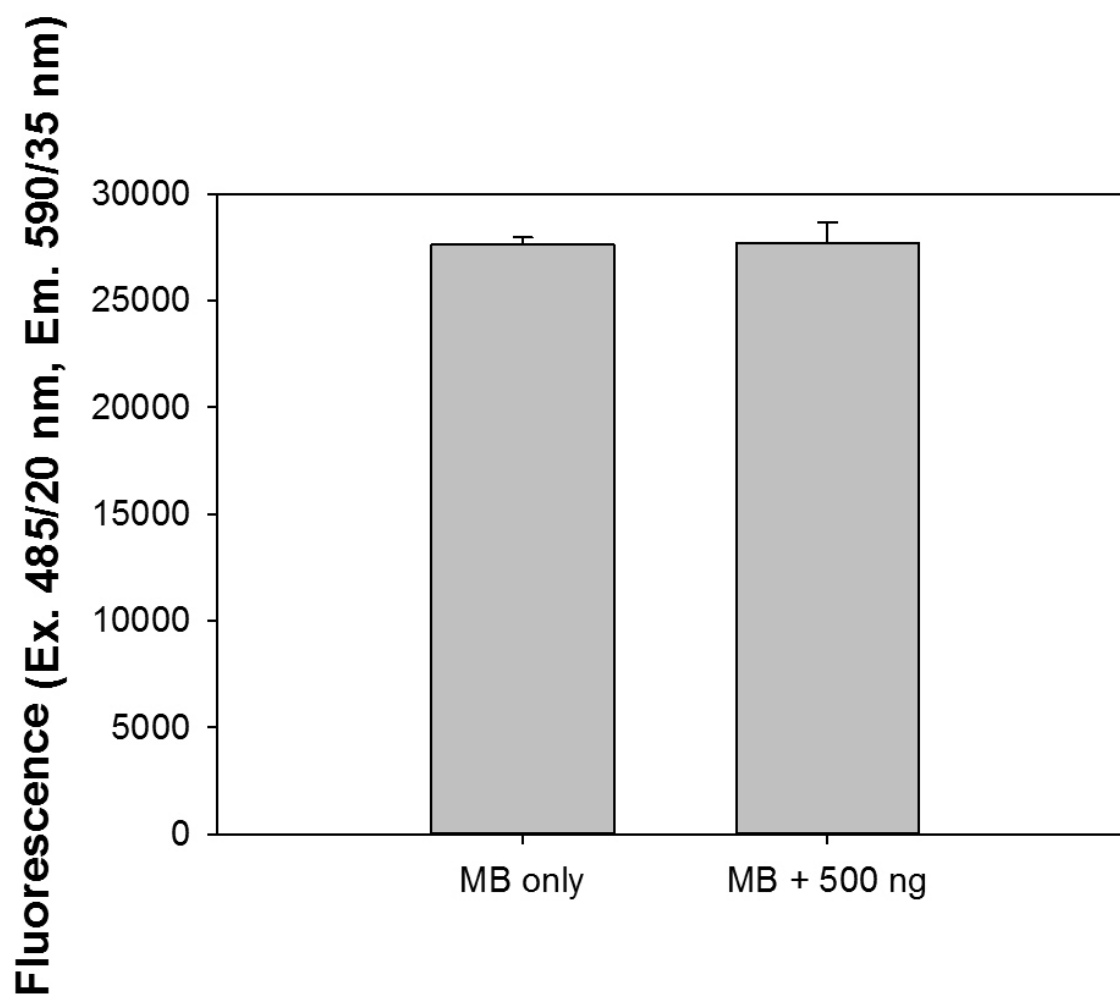


Figure 6. Fluorescence of 8 nM of MB (*Bt cry1Ac*) designed for detecting the *Bt cry1Ac* gene in the presence of bacterial (*Bacillus thuringiensis*) genomic DNA (500 ng) in chicken broth (100%) using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 75. Error bars report standard error.

**Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013**

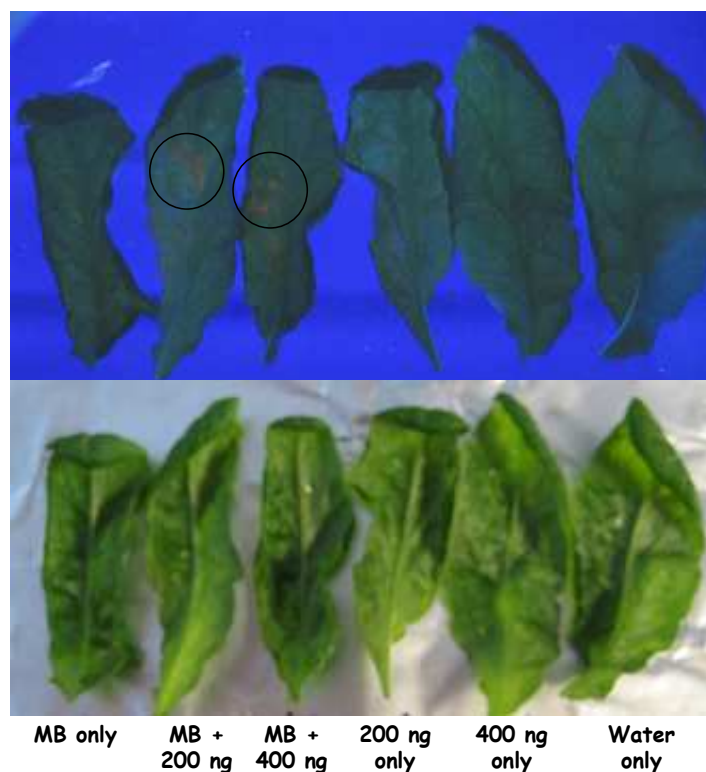


Figure 7. Arabidopsis as a model system for detecting *Bacillus thuringiensis*. Fluorescence of 40 nM of molecular beacon (*Bt cryIAc*) designed for detecting *Bt cryIAc* gene in the presence of bacterial (*B. thuringiensis*) genomic DNA co-infiltrated into Arabidopsis plants observed in MB only, MB in the presence of 200 ng or 400 ng bacterial genomic DNA, 200 ng or 400 ng bacterial genomic DNA alone and water only under 302 nm UV light (above) and white light (below).

Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013

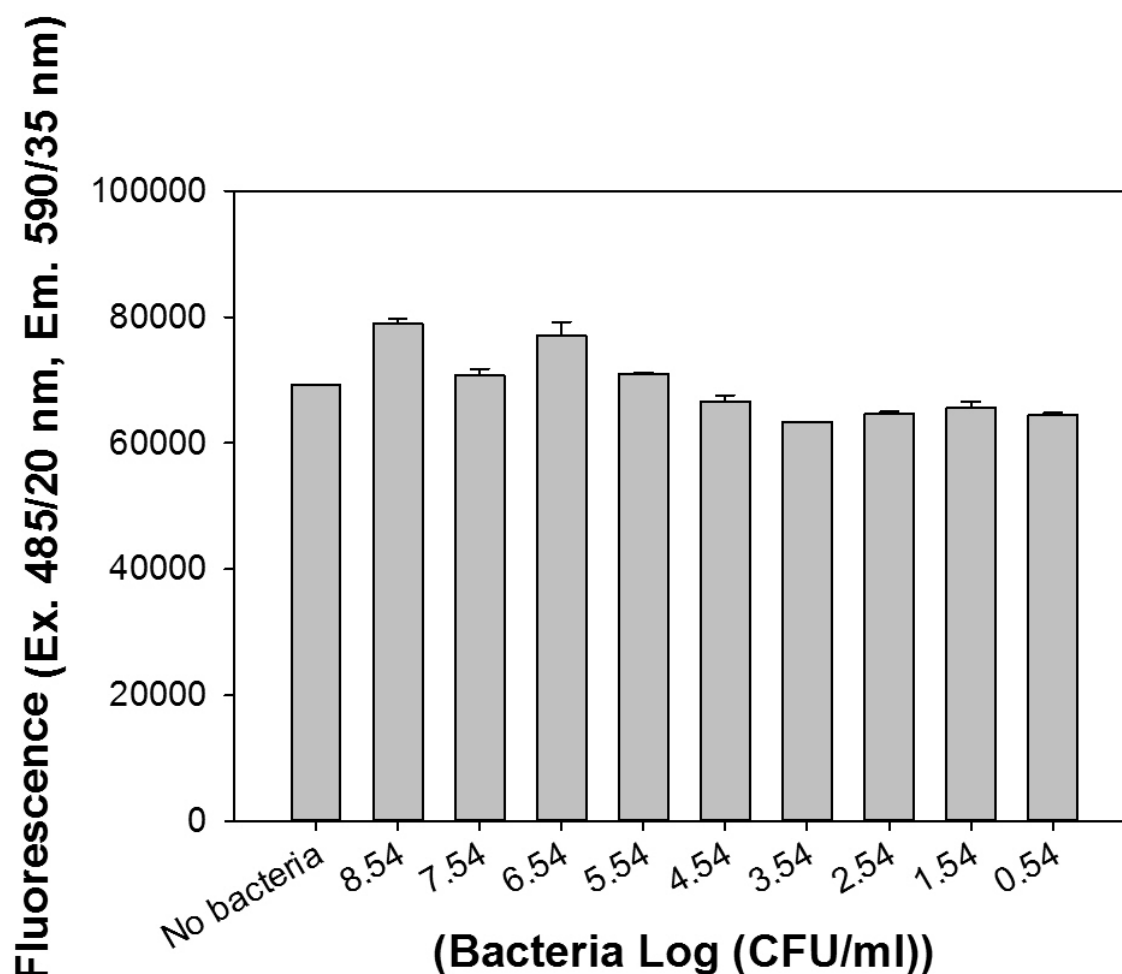


Figure 8. Fluorescence of 3.3 nM of MB (*Bt cry1Ac*) designed for detecting the *Bt cry1Ac* gene in the presence of bacterial (*Bacillus thuringiensis*) genomic DNA (using GenElute™ Bacterial Genomic DNA kit (SigmaAldrich, St. Louis, MO) following manufacturer's instructions) (diluted 10^0 to 10^{-8}) using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Genomic DNA concentrations were determined to be 125 ng/μl to 5 ng/μl from 8.54 log CFU/ml to 0.54 log CFU/ml. The limit of detection was determined to be 5.54 log CFU/ml, based on observed fluorescence. Error bars report standard error.

Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013

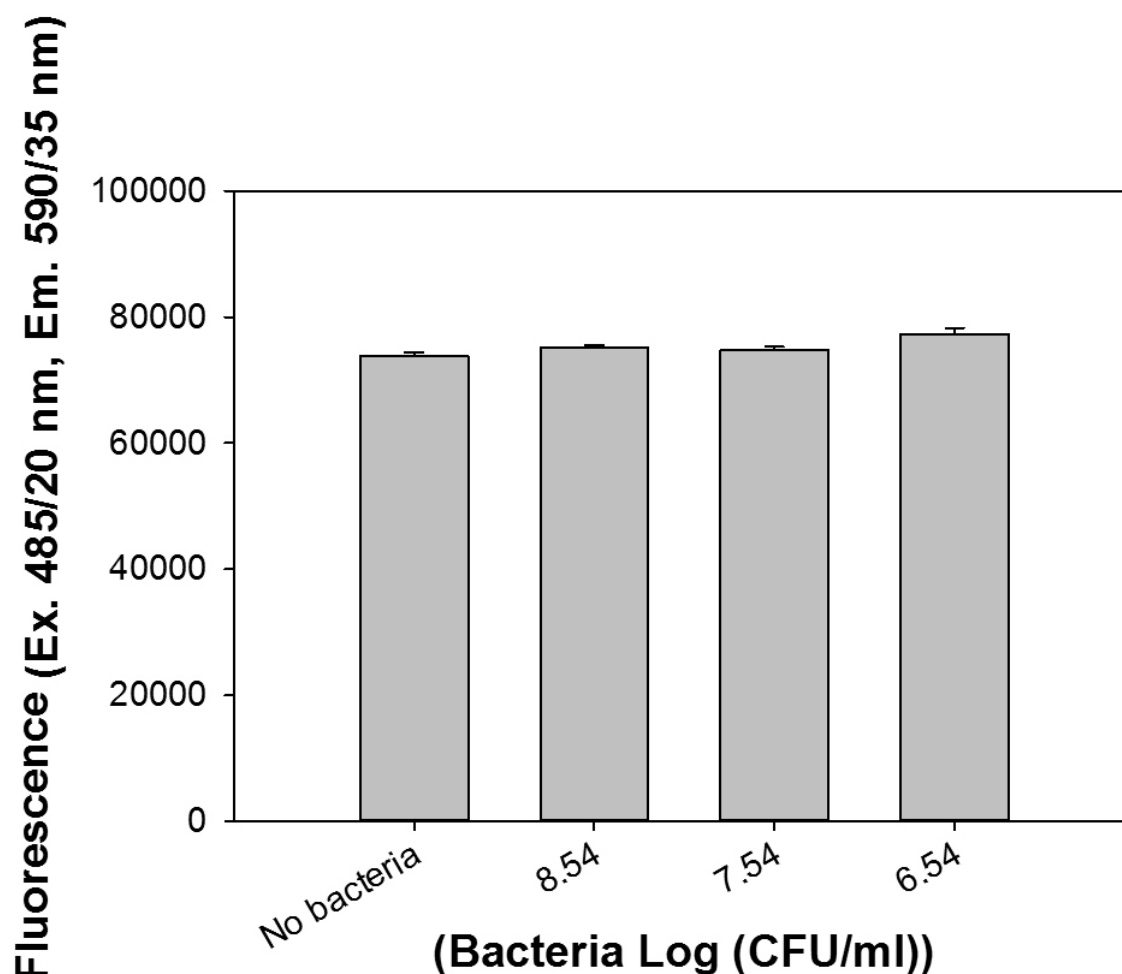


Figure 9. Fluorescence of 3.3 nM of MB (*Bt cry1Ac*) designed for detecting the *Bt cry1Ac* gene in the presence of bacterial (*Bacillus thuringiensis*) genomic DNA (using the Goldenberger et al. (1995) protocol) (diluted 10^0 to 10^{-2}) using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Genomic DNA concentrations were determined to be 1621 ng/ μ l to 17 ng/ μ l from 8.54 log CFU/ml to 6.54 log CFU/ml. The limit of detection was determined to be 5.54 log CFU/ml (the lowest log CFU/ml tested). Error bars report standard error.

Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013

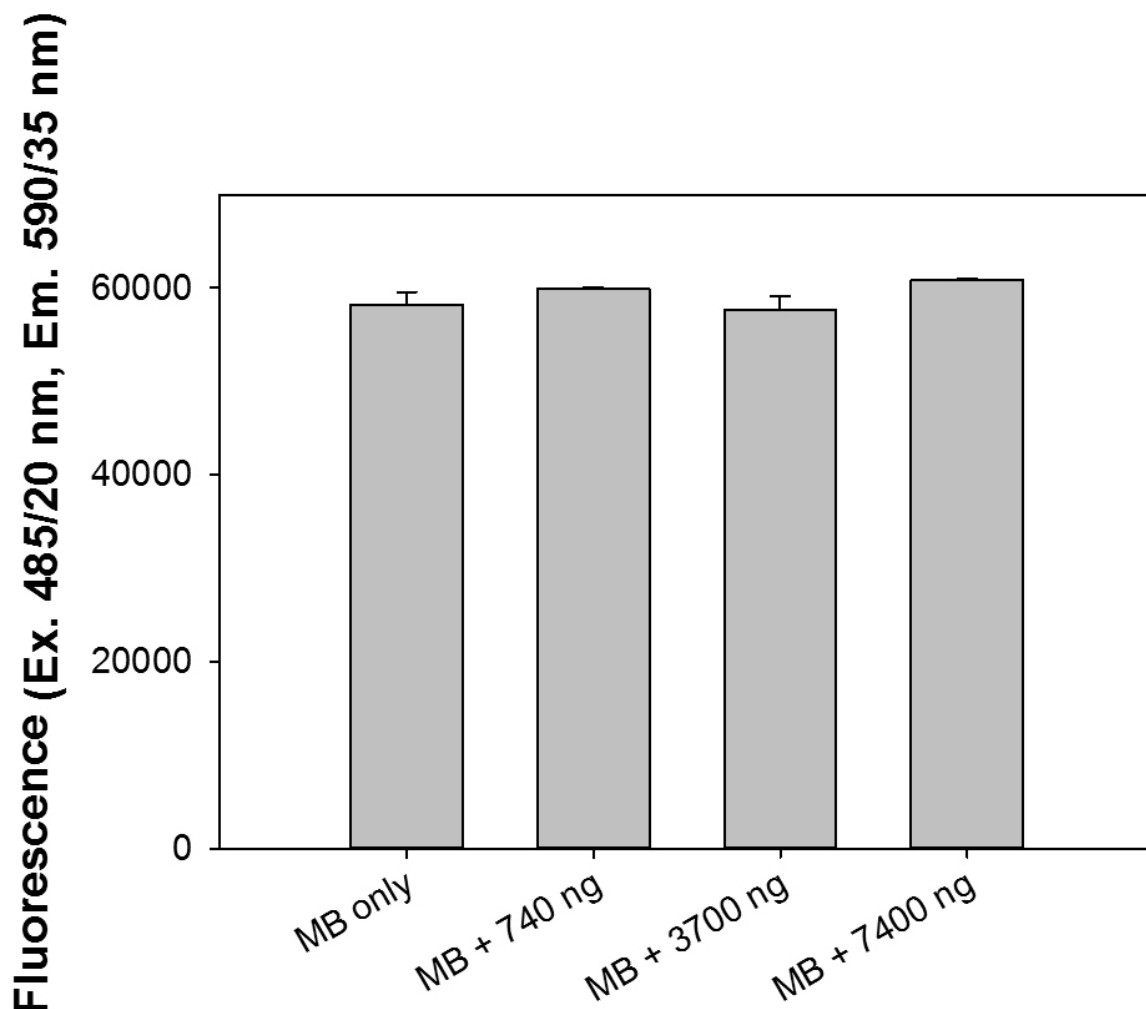


Figure 10. Fluorescence of 33 nM of MB (*Bt cryIAc*) designed for detecting the *Bt cryIAc* gene in the presence of plant (transgenic tobacco) genomic DNA (740, 3700 or 7400 ng) in water using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100. Error bars report standard error.

**Scientific Progress and Accomplishments for Report period May 1, 2008
through March 31, 2013**

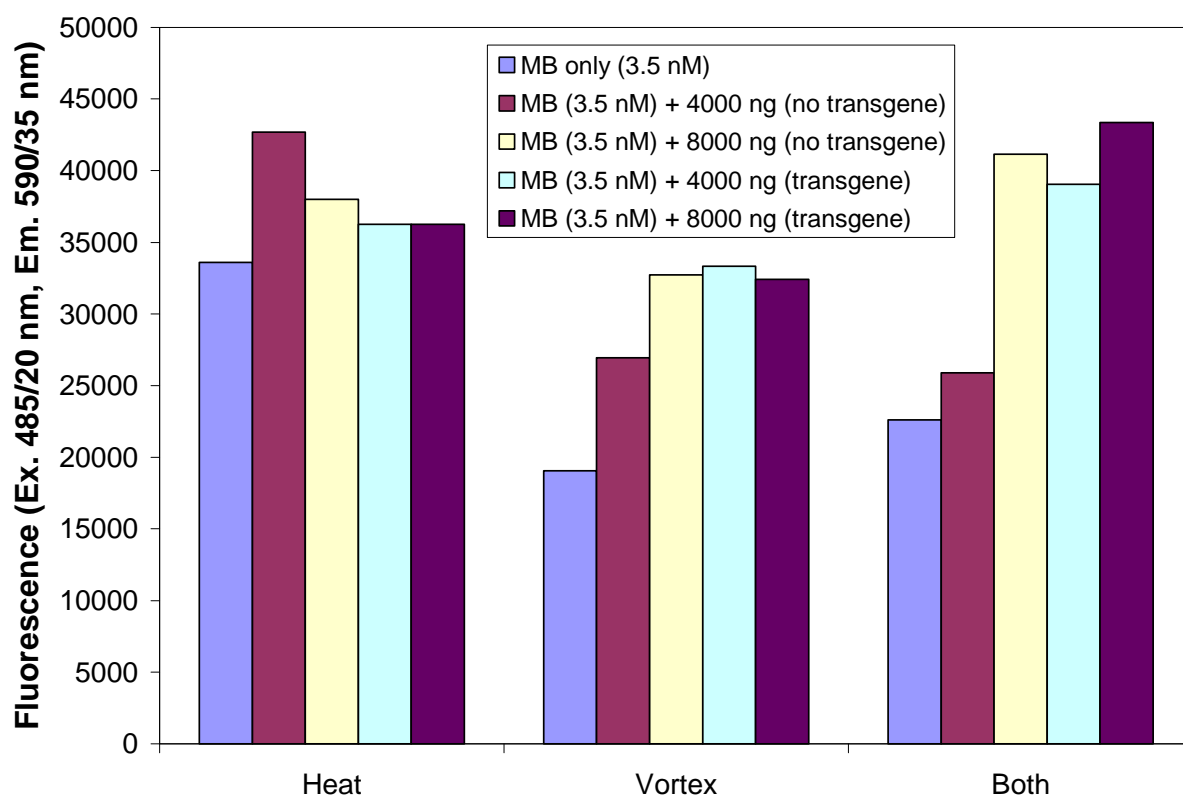


Figure 11. Fluorescence of 3.5 nM of MB (*Bt cryIAc*) designed for detecting the *Bt cryIAc* gene in the presence of plant (transgenic or non-transgenic tobacco) genomic DNA (4000 or 8000 ng) treated (heating at 95 °C for 5 min, vortex for 15 sec, or both (heating at 95 °C then vortexing for 15 sec)) using excitation wavelength of 485/20 nm and emission wavelength of 590/35 nm with sensitivity at 100.